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The interaction between a normal cell and its substratum, a phenomenon known as anchorage dependence, is an important determinant of the G_1/S transition. The cell surface receptors that mediate cell-substrate adhesion are known as integrins. It is possible that extracellular matrix proteins regulate normal cell growth by transmitting signals to the interior via specific integrin receptors. In transformed cells such signaling pathways might be absent or dysfunctional thereby conferring unresponsiveness to normal growth constraints. The present studies are focused to understand how integrin receptors, in particular $\alpha 5\beta 1$, participate in the regulation of cell division in normal breast cells and to determine how breast cancer cells escape these regulatory pathways. Our preliminary findings demonstrate that ligation of the $\alpha 5\beta 1$ integrin receptor in normal breast cells by GRGDS peptides results in the rapid activation of cdc2 a cyclin dependent kinase active at the G_1/S transition. These findings provide the first clear evidence in support of a direct role for integrin receptors in the control of normal cell division. The effects of $\alpha 5\beta 1$ mediated signaling in transformed BC will be investigated.

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Elizabeth A. Wayner   10/25/95
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RESEARCH GRANT DAMD 17-94-J-4303
PRINCIPAL INVESTIGATOR: ELIZABETH A. WAYNER

YEAR 1 PROGRESS REPORT: OCTOBER 29, 1995

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RESEARCH GRANT DAMD 17-94-J-4303
PRINCIPAL INVESTIGATOR: ELIZABETH A. WAYNER

YEAR 1 PROGRESS REPORT: OCTOBER 29, 1995

NEW PRINCIPAL INVESTIGATOR STATUS

The grant #DAMD 17-94-J-4303 entitled "Mechanisms of Integrin-Mediated Growth Control in Normal, Transformed, and Neoplastic Breast Cells" was awarded to Dr. Banu Symington in September, 1994. Dr. Symington has returned to private practice and I have been appointed as the Principal Investigator so that these important studies can be continued (Notice of Grant Award, July 1995). I am very enthusiastic about this project and despite a delay in transferring the grant and initiating the project we have made substantial progress since July, 1995. Therefore, I do not anticipate any difficulties in completing the proposed studies within a four year grant period. Because of the delay in getting started and to ensure a four year grant period I found it necessary to request a one year no cost extension.

ALTERATIONS IN PERSONNEL AND EXPENDITURES

PERSONNEL

1. <u>Elizabeth A. Wayner, Ph.D.</u> (Principal Investigator). I am a Senior Scientist at the Seattle Biomedical Research Institute and an Associate Professor in the Department of Pathobiology at the University of Washington. I have extensive training and experience in cell biology, immunology and the biochemistry of cell adhesion molecules and have published widely in these fields. I will be responsible for the coordination of all aspects of the proposed research as well as performance of many of the studies including data analysis and publication, isolation and growth of breast cancer cells *in vitro*, examining the regulation of CDK/cyclin complex formation in breast cells as a function of integrin expression and activity, and monoclonal antibody production. The SBRI is fully equipped with a wide range of equipment necessary to complete all of the work described in the proposal. Therefore, I do not anticipate any difficulties in completing the proposed studies in my present environment. In order to ensure the successful initiation of the proposed studies I have substantially increased my percent effort and I will adjust the percent effort contribution of my support staff accordingly. 50% of my salary was encumbered on this grant as of August 1, 1995 (see the financial report from SBRI in Appendix).

2. <u>Tom Burkett</u> (Postdoctoral Fellow). Dr. Burkett is a highly trained molecular geneticist with experience with both cell cycle regulation in yeast and the molecular cloning and production of bivalent antibody reagents. He will be responsible for the isolation and characterization of normal and neoplastic breast cell lines and will devote a significant portion of his effort to the studies described in Aims 1-3. I anticipate that Dr. Burkett will begin working in my laboratory in early November, 1995. A copy of Dr. Burkett's curriculum vitae is in the Appendix.

3. <u>John Fisher</u> (Research Technician II). John will be responsible for obtaining the surgical specimens from Dr. Tickman's laboratory and for the culture of normal and transformed breast cell lines. His duties will also include maintenance of anti-integrin monoclonal hybridoma cell lines, routine isolation of normal breast cells, preparation of buffers, data recording and analysis, routine laboratory maintenance and ordering. John has already made significant progress in assisting me to establish my laboratory and his effort will be absolutely essential for the completion of the proposed studies. His salary has been encumbered on this grant since August 1, 1995 and he will devote 100% of his effort to this project.

EQUIPMENT, CONSUMABLE SUPPLIES, TRAVEL, PUBLICATION, OTHER

We do not anticipate changes in the budget for expenditures other than salary.

A. INTRODUCTION

1. Nature of the Problem

Breast cancer is the leading cause of cancer-related death among women in this country and the incidence of breast cancer among young women is rising. Although early detection techniques (mammography) are available, early detection does not necessarily predict survival and cannot be used to prevent or treat breast cancer. Developing new strategies to improve breast cancer survival, therefore, will depend upon our greater understanding of the pathogenesis of breast cancer and the events which lead to neoplastic transformation of breast epithelial cells.

The hallmark characteristics of malignant transformation include uncontrolled or anchorage-independent growth, local invasion of adjacent tissues and the acquired ability to invade blood vessels and "home" to sites distal from the primary tumor (metastasis). An important aspect of tumor invasion and metastasis involves the adhesive interactions of tumor cells with other cells or the extracellular matrix. Many of these adhesive interactions are mediated by the integrin family of cell surface receptors (reviewed by Hynes, 1992). However, it has become increasingly clear that integrins also participate in the development of anchorage-independent growth since transformed cells do not require substrate attachment to proliferate. It is possible that extracellular matrix proteins regulate normal cell growth by transmitting signals to the interior via specific integrin receptors. In transformed cells such signaling pathways might be absent or dysfunctional thereby conferring unresponsiveness to normal growth constraints. **Our efforts are focused to understand how integrin receptors, in particular $\alpha5\beta1$, participate in the regulation of cell division in normal breast cells and to determine how breast cancer cells escape these regulatory pathways.**

2. Background of Previous Work

Integrin Receptors. Integrins are transmembrane glycoproteins composed of two non-covalently associated subunits (α and β) that mediate both cell-cell and cell-substrate adhesion. Integrin receptors bind extracellular matrix (ECM) and plasma proteins, non-integrin adhesion receptors and other integrins (reviewed by Hemler, 1990; Hynes, 1992). In addition to their role as primary mediators of cell adhesion, it has now become clear that integrins are also capable of transducing signals to the cell interior (reviewed by Ginsberg et al., 1992, Juliano, 1993; Juliano, 1994). Tyrosine phosphorylation appears to be a key aspect of integrin mediated signal transduction and a tyrosine kinase ($p125^{FAK}$) has been identified which localizes to focal adhesions when cells are plated on adherent protein coated substrata (Schaller et al., 1992). Furthermore, evidence is accumulating which suggests that integrin-mediated signaling events can induce gene expression (Yurochko et al., 1992) and affect transit through the cell cycle (Matsuyama et al., 1989; Symington, 1992; Mortarini et al., 1992). Many of the ECM proteins that serve as ligands for integrins have been identified and include: fibronectin (FN), vitronectin (VN) the laminins, and collagens. Some integrins can interact with more than one ligand (e.g. $\alpha4\beta1$, $\alpha v\beta3$) while several ECM proteins have been shown to engage more than one integrin. For example, FN has been shown to interact with multiple integrin receptors (e.g. $\alpha v\beta3$, $\alpha4\beta1$, $\alpha5\beta1$) and certain integrins (e.g. $\alpha v\beta3$) can recognize a specific peptide sequence (arginine-glycine-aspartic acid, RGD) common to many ECM proteins. An exception to this is the $\alpha5\beta1$ receptor, which appears to bind solely to the GRGDS sequence located in the central cell binding domain of fibronectin (Ruoslahti, 1988).

Integrins and Cancer. In order to understand the role of integrins in tumor cell biology it is important to have information concerning the pattern of integrin expression in transformed cells and to ask whether there is any relationship between integrin expression and tumor progression. It has long been known that transformed fibroblasts express less fibronectin than normal controls with reduced cell surface fibronectin contributing to altered morphology, adhesiveness, and motility (Yamada, et al., 1976; Yamada, 1983). Changes in integrin expression have been observed in a variety of cultured malignant cells (Wayner et al., 1988) and it has been well documented that chemical or viral transformation can affect both the expression and function of numerous integrin receptors (Plantefarber and Hynes, 1989; Akiyama et al., 1990; Dedhar and Saulnier, 1990). Other in vitro studies have revealed that increased expression of $\alpha5\beta1$ is negatively correlated with transformation (Giancotti and Ruoslahti, 1990; Symington, 1990; Schreiner et al., 1991; Varner et al., 1992) while increased expression of another integrin receptor ($\alpha v\beta3$) is positively correlated with

transformation (Albeda et al., 1990; Fielding-Habermann et al., 1992; Gehlsen et al., 1992). These in vitro data are consistent with the results of immunohistochemical studies performed with a variety of carcinomas which have revealed a strong correlation between decreased expression of α5β1 and increased tumor progression (Stallmach et al., 1992; Weinel et al., 1992). **Together these findings strongly support the hypothesis that expression of α5β1 is associated with a less malignant phenotype.**

Integrins and Breast Cancer. Normal breast cells express a variety of integrin receptors including α2β1, α5β1, avβ5, α3β1 and α6β4. However, neoplastic breast cells have been reported to express reduced levels of α5β1, α6β4 and α2β1 (Zutter al., 1990 Jones et al., 1992; Pignatelli et al., 1992) and increased levels of α3 (Miettien et al., 1993). In addition, epithelial tumors in general consistently display alterations in the spatial organization of integrins in contrast to neighboring normal cells. For example, in normal epithelia the α3, α6 and β4 receptors are localized to the lateral (α3) and basolateral (α3, α6 and β4) surface in close proximity to the basement membrane consistent with their role in mediating attachment to the basement membrane (Carter et al., 1991; Jones, 1991; Sonnenberg et al., 1991). This organization is completely disrupted in cutaneous (Sollberg et al., 1992) or breast (Natali et al., 1992; Pignatelli et al., 1992) carcinomas. In addition, metastatic breast cells often exhibit altered integrin expression when compared to cells derived from the primary tumor (Natali et al., 1992; Gui et al., 1995). Recently, Zutter et al. (1995) have shown that transfection of α2β1 into a highly metastatic α2β1 negative breast carcinoma cell line resulted in the acquisition of a more normal phenotype, anchorage-dependent growth and a reduced ability to form tumors in vivo strongly implicating the α2β1 integrin receptor in the control of breast cancer cell behavior. The studies described in this proposal, however, will concentrate on evaluating the role of altered α5β1 function in the control of tumor growth and the progression of breast cancer.

Cell Cycle Regulation. In normal eukaryotic cells the transitions between different stages of the cell cycle are tightly regulated at "checkpoints" (Nurse, 1994). Transitions through key checkpoints are under the control of a unique family of protein kinases, the cyclin-dependent kinases or CDKs (Nurse, 1990; Hartwell, 1991). The expression of cyclins in normal cells is scheduled, occurring at specific phases of the cell cycle while CDKs are stably expressed throughout the cell cycle. At each checkpoint point certain cyclins and CDKs form active complexes which phosphorylate and thereby activate specific proteins necessary for DNA replication (G_1/S), mitosis and cytokinesis (G_2/M). Recently, it has been shown that the activity of cyclin/CDK complexes is regulated by a family of CDK inhibitor proteins or CDIs (p16, p21, p27) that bind to and inactivate the CDKs (Hunter and Pines, 1994).

Transition through the cell cycle is dependent upon both positive and negative extracellular growth signals. In eukaryotic cells, these signals have the most profound effect on the G_1 to S phase transition. In *Saccharomyces cerevisiae* mating pheromones, cell size and nutrient abundance all influence cell cycle progression at a point in G_1 called "start". In mammalian cells the "restriction point" is an analogous event and the predominant effectors of the G_1/S transition are growth factors, growth inhibitory proteins (CDIs), cell-cell interactions and cell-substratum interactions. Pathways linking extracellular signals to cell cycle progression can target CDK activity; growth promoting signals induce CDK activation while growth inhibitory signals down regulate the CDKs. Extracellular signals can also regulate cell cycle progression by targeting CDIs and can either promote the G_1/S transition by inactivating a specific CDI, or such signals could arrest cells in G_1 by inducing CDI expression and consequently inactivation of specific G_1 cyclin/CDK complexes. For example, IL-2 has been reported to promote T lymphocyte proliferation by inactivation of a CDK2 inhibitor (Firpo et al., 1994), while mammalian cells arrested in G_1 by TGF-β express elevated levels of p27 which inhibits the formation of catalytically active cyclin/cdk2 complexes (Polyak et al., 1994). The known tumor suppresser factors, retinoblastoma protein (Rb) and p53, are also involved in cell cycle regulation and can interact with either G_1/S cyclins (phosphorylated Rb) or CDIs (p53 induces p21). Integrin receptors could contribute to the regulation of cell division in several ways; signals transduced through integrins could initiate mitosis by providing a "start" or mitogenic signal, or integrin receptors could negatively regulate cell division via the induction of a specific inhibitor such as p21, p16 or p27. In this regard, it is interesting that p27 is induced by cell-cell contact (Polyak et al., 1994) suggesting that integrins might participate in the return to homeostasis (G_0) via a p27 dependent mechanism activated by cell contact. **The purpose of the present studies is to determine how signals transduced via integrin receptors (α5β1) regulate the formation and activity of cdk/cyclin complexes in neoplastic and normal breast cells.**

Role of Integrins in Cell Cycle Regulation. The results of several studies support a role for integrins, in particular α5β1, in the control of normal cell growth. The interaction between a normal cell and its

substratum, a phenomenon known as anchorage dependence, is an important determinant of the G_1/S transition. It has been proposed that regulated cyclin expression may play a major role in anchorage-dependent proliferation (Elledge et al., 1992). It now appears that cyclin A expression is one component of the anchorage requirement and that deregulating cyclin A synthesis could be involved in the development of anchorage-independent growth by transformed cells. Anchorage-dependent cells placed into suspension fail to express cyclin A and ectopic expression of cyclin A but not other cyclins was sufficient to override the anchorage requirement (Guadagno et al., 1993). This suggests that in normal cells signaling through an integrin receptor might trigger cyclin A transcription during late G_1, and that the requirement for this signal might be bypassed during transformation. Matsuyama et al., 1989 showed that stimulation by solid phase anti-CD3 was insufficient to activate proliferation of CD4+ T lymphocytes. Such cells, when cultured in serum-free medium (no fibronectin) required ligation of the α5β1 FN receptor and CD3 for proliferation to occur. In another study, Yamada et al., 1991 showed that anti-CD3 or fibronectin alone were insufficient to induce IL-2 message by CD4+ T cells. Induction of IL-2 message in CD4+ T cells required the concomitant ligation of both CD3 and α5β1. Importantly, these workers went on to show that ligation of α5β1 in CD4+ T cells induced expression of an AP-1 transcription factor. These studies demonstrate clearly that in T lymphocytes α5β1 can cooperate with other cell surface receptors (e.g. CD3) to induce signals which trigger proliferation. Other workers have shown that wounding of epithelial tissues results in increased α5β1 expression and proliferation (Hertle, et al., 1991; Pellegrini et al., 1992; Grinnell, 1992; Pujades et al., 1992) while Mortarini et al. (1992) have shown that quiescent melanoma cells cultured in serum free medium proliferated in a dose and time dependent fashion when exposed to immobilized fibronectin. Finally, the results of several studies have shown that over expression of α5β1 in various tumor models restores anchorage dependent growth and reduces the ability of such cells to grow in soft agar or to form tumors in nude mice (Giancotti and Ruoslahti, 1990; Symington, 1990; Schreiner et al., 1991). **These findings, together with the historical data demonstrating that FN can reverse certain abnormalities observed in transformed cells (reviewed by Ruoslahti, 1988) strongly support the hypothesis that α5β1 directly regulates cell growth.**

3. Work Carried Out By Previous Investigator (Dr. Banu Symington)

Work Accomplished with FA-K562 Cells (Symington, 1990; 1992). FA-K562 cells were selected for their ability to adhere to fibronectin and over express α5β1 relative to the original parent population (K562). FA-K562 cells proliferated more slowly than K562 cells in liquid culture, had a reduced plating efficiency in soft agar and were non-tumorigenic in nude mice. Treatment of FA-K562 cells with GRGDS peptide or an anti-α5 Mab (P1D6) induced growth in soft agar strongly suggesting that signals transduced via α5β1 were linked to soft agar growth. In subsequent experiments it was determined that exposing FA-K562 cells to 10 ug/ml GRGDS for 2 hrs specifically stimulated p34/cdc2 and cyclin A associated kinase activities. DNA staining profiles demonstrated that GRGDS induced cell cycle progression within 24 hr. The 110 kDa form of the retinoblastoma (Rb) protein appeared within 3 hrs of GRGDS addition, consistent with the activation of a G1/S kinase. The effect of GRGDS could be inhibited by Mabs directed to the α5 (P1D6) or β1 (P4C10) integrin receptors. Control peptide, GRGES, which cannot bind α5β1 did not induce proliferation or cdc2 activation. **The results of these studies strongly suggest that in FA-K562 cells, α5β1 and its extracellular ligand GRGDS can regulate cell proliferation.**

GRGDS Stimulates Keratinocyte cdc2 Activity and Proliferation. FA-K562 cells are derived from an erythroleukemia cell line, K562, and are therefore not closely related to breast epithelial cells. Therefore, the effects of α5β1 ligation with GRGDS were examined in primary cultures of keratinocytes derived from neonatal foreskins. FN and GRGDS both stimulated cdc2 activity and proliferation of normal human keratinocytes. No other integrin ligand stimulated keratinocyte proliferation. These studies show that GRGDS-responsive growth pathways exist in normal primary epithelial cells as well as in cultured leukemic cells and that these pathways are not dependent on overexpression of α5β1 since primary epithelial cells express low levels of α5β1. Next, the ability of GRGDS to stimulate cdc2 activation in transformed keratinocytes was examined. The EIL8 and 1811-T1 cell lines were derived by human papilloma virus-transfection of normal keratinocytes (Kaur and McDougall, 1989; Kaur and Carter, 1992; Symington et al., 1993) and retain many of the properties of keratinocytes. However, only 1811-T1 cells exhibit anchorage-independent growth in soft agar and can form tumors. Preliminary studies demonstrated that cdc2 activation

by GRGDS occurred in the partially transformed EIL8 cells but was impaired in the tumorigenic 1811-T1 cells. **The results of this study show that ligation of $\alpha5\beta1$ in normal or transformed but not tumorigenic keratinocytes can promote cdc2 activation and proliferation.**

Effects of $\alpha5\beta1$ Ligands on Breast Cell Growth. GRGDS could stimulate cdc2 kinase activity in a transformed but not tumorigenic breast cell line (HBL-100), but not in a fully transformed breast cell line (BT20). In addition, HBL-100 cells were selectively induced to proliferate by $\alpha5\beta1$ ligands but not by ligands recognized by integrin receptors such as VN, LM, or COL. In contrast, BT20 cells were not induced to proliferate by FN or GRGDS. **These results show clearly that cdc2 activity in transformed but not tumorigenic breast carcinoma cells could be regulated by $\alpha5\beta1$.**

4. Purpose of the Present Work

Hypotheses. The results of the studies carried out by the previous PI led to the following important conclusions. First, that $\alpha5\beta1$ signals growth responses in normal or transformed but not tumorigenic keratinocytes or breast cells. Second, that signals delivered through $\alpha5\beta1$ lead to activation of cdc2 and consequently to proliferation. Third, that transformation of breast cells, similar to keratinocytes, promotes loss of normal growth responses to $\alpha5\beta1$ ligands. Fourth, the data also suggest that as cells become more phenotypically abnormal they experience a progressive reduction in the ability to be regulated by GRGDS and $\alpha5\beta1$. Together these findings suggests that escape from GRGDS-responsive growth is a common feature of transformation. The purpose of the present work is two-fold. First, the molecular mechanism of $\alpha5\beta1$ induced proliferation in normal breast cells will be determined. Second, the mechanism(s) by which breast cancer cells escape these normal growth regulatory pathways will also be determined. Three hypotheses will be tested.

1. Ligation of $\alpha5\beta1$ with GRGDS peptides or anti-$\alpha5$ Mabs will trigger cell division in normal but not neoplastic breast cells.

2. Escape from $\alpha5\beta1$-mediated growth signaling is a key step in neoplastic breast cell transformation.

3. $\alpha5\beta1$ signaling can be used to either predict tumor outcome or be useful as an early diagnostic test for progressive or metastatic tumors.

5. Methods of Approach

> **New Technical Objective (Aims 2 and 3): Identify and characterize the integrin receptors expressed by freshly derived and cultured neoplastic breast cells (BC).**

We have added this Technical Objective which will be incorporated into Aims 2 and 3 for the following reasons. We have discovered that the integrin phenotype and consequently the function of integrin receptors expressed by breast cell lines varies considerably (see Results Section). In particular, some cultured breast carcinoma cell lines express another high affinity FN receptor, $\alpha v\beta3$, which is also capable of interacting with RGD-containing peptides (reviewed by Hynes, 1992; Fielding-Habermann and Cheresh, 1993). Furthermore, we have shown that in carcinoma cell lines which express both $\alpha v\beta3$ and $\alpha5\beta1$, $\alpha v\beta3$ is the predominant receptor involved in binding to fibronectin (Wayner et al., 1991; Leavesley et al., 1992). In view of these findings and the potential role $\alpha v\beta3$ may have in determining tumor cell behavior (Fielding-Habermann and Cheresh, 1993), it will be important to screen all cultured cell lines and primary breast cell populations for the presence of this receptor. Studies designed to identify and probe the adhesive function of BC RGD-interactive integrin receptors other than $\alpha5\beta1$ were not included in the original grant application. These experiments will now be incorporated into Aims 2 and 3 and cell lines or freshly isolated BC populations which express alternative RGD-interactive receptors, in particular $\alpha v\beta3$, will be excluded. If time and funds permit we may eventually investigate $\alpha v\beta3$ (or other integrin) dependent cell cycle events. Text pertaining to alterations in the original Technical Objectives (Aims) is boxed (see below).

> **New Technical Objective Incorporated into Aims 2 and 3: Identify and characterize the integrin receptors expressed by freshly derived and cultured neoplastic breast cells (BC).**
>
> a) The expression of integrin receptors by BC will be examined by flow cytometry analysis and by standard protein chemistry techniques (immunoprecipitation).
>
> b) The function of integrin receptors expressed by BC will be examined using a standard cell adhesion assay and well characterized function inhibiting Mab reagents.

B. BODY

1. Experimental Methods

a. Patient Samples

Freshly isolated BC needed for Aim 3 will be obtained from lumpectomy and mastectomy tissues provided by Dr. Ron Tickman who is a staff pathologist in the Laboratory of Pathology, Swedish Hospital Medical Center, Seattle, WA (see letter from Dr. Tickman in the Appendix). Based on my conversations with Dr. Tickman I anticipate obtaining at least two cancer or normal breast specimens per week. These samples will be removed from patients undergoing surgery at Swedish Hospital solely for diagnostic or therapeutic purposes. The samples would otherwise be discarded and information identifying the patients will be withheld in order to comply with the requirements to obtain exemption status from complete IRB review. However, I will have access to information concerning the patient's age and sex, tumor histology, estrogen and progesterone receptor status, and the presence and location of metastases. This will enable me to correlate tumor type, malignancy, integrin expression and response to $\alpha5\beta1$ ligation in the studies described in Aim 3. Although no patient samples will be excluded on the basis of race, age, sex, religion, or ethnic background, the low incidence of male breast cancer will preclude collection of a large number of samples from men. I have received approval for the use of these tissues from the IRB at SBRI.

b. Monoclonal and Polyclonal Antibodies

Anti-integrin Monoclonal Antibodies. My laboratory has produced monoclonal antibodies (Mabs) directed to a variety of integrin receptors expressed by normal and neoplastic epithelial cells (Wayner et al., 1987; Wayner et al., 1988; Carter et al., 1990a; 1990b, Brown et al., 1991; Wayner et al., 1991; Wayner et al., 1993). Many of these Mabs perturb integrin function and will be used to determine how normal and neoplastic breast cells interact with FN or GRGDS peptide. The two anti-integrin Mab reagents used by Dr. Symington to ligate $\alpha5$ or $\beta1$ were made in my laboratory, P1D6 (anti-$\alpha5$) and P4C10 (anti-$\beta1$) and I have unlimited quantities available for the present studies. Other hybridoma cell lines which secrete inhibitory anti-integrin Mabs and are available in my laboratory for use in the present studies include: P1H5 (anti-$\alpha2$), P1B5 ($\alpha3$), P4C2 ($\alpha4$), P5H9 ($\alpha v\beta5$), and LM609 ($\alpha v\beta3$, courtesy of Dr. David Cheresh, Scripp's Institute, La Jolla, CA). **Antibodies Directed to Human Cell Cycle Proteins.** Monoclonal or polyclonal antibodies directed to human CDKs (cdc2, cdk2-cdk5), cyclins (A, B1, D1-D3, E), CDIs (p16, p21, p27), and tumor suppresser proteins (Rb and p53) will be obtained from Pharmingen (1995 Catalogue and described in Hot Lines, 2[1]: 3-4) or from Dr. Jim Roberts, Fred Hutchinson, Seattle, WA (letter provided upon request).

c. Breast Cell Isolation and Culture

Normal human BC will be obtained from Clonetics Corp. (San Diego, CA, cat. #CC-0228) or from reduction mammaplasty tissue supplied by Dr. Ron Tickman (see letter in the Appendix). Neoplastic breast epithelial cells will be obtained from tissues supplied by Dr. Tickman. Breast epithelial cells will be isolated according to published protocols (Smith and Hackett, 1987; Band and Sager, 1989). The tissues will be minced and enzymatically digested to derive single cells. Breast cells will be plated in serum-free mammary epithelial cell growth medium (MEGM) from Clonetics (cat. #CC-3051) to establish cell lines, or they will be used immediately. The techniques used to obtain primary breast epithelial cells are similar to those I have used to obtain primary human epidermal keratinocytes (Carter et al., 1990a; 1990b; Wayner et. al, 1993) therefore, we do not anticipate problems with this approach.

d. Breast Cell Proliferation Assay

Exponentially growing BC will be trypsinized and seeded at 5×10^3 cells per well in 96 well flat bottomed microtest plates in complete MEGM. In some experiments (Aim 3) freshly derived BC will be seeded into microtest plates. Plating densities for freshly isolated BC will be determined by the efficiency of the isolation procedure. Serum free medium will be used to avoid complications due to integrin interactive ligands in serum. After 24 hrs the cultures will be stimulated with $\alpha5\beta1$ interactive ligands or anti-$\alpha5$ or anti-$\beta1$ Mabs. At intervals, cell numbers will be estimated by fixing the cells, staining them with crystal violet, solubilizing and evaluating absorbency at 540 nm (the intensity of the staining is proportional to cell number). Alternatively, at intervals, the cultures will be pulsed with 1 uCi of [methyl-^{3}H]thymidine (New Enlgand Nuclear) for 4 hrs and harvested on an automatic cell harvester (Skatron) for scintillation counting. Tritiated thymidine incorporation might prove to be a more sensitive method to detect the effects of $\alpha5\beta1$ ligation on initiating the G_1/S transition in limiting numbers of fresh breast cells.

e. Inhibition of Cell-Substrate Adhesion with Anti-Integrin Mabs

Inhibition of BC adhesion to various ligands will be performed essentially as described (Wayner and Carter, 1987; Wayner et al., 1989; Wayner et al., 1991). Briefly, 48 or 96 well virgin styrene plates will be coated with adhesive ligands (5-10 ug/ml). The plates will be blocked with PBS supplemented with 10 mg/ml heat denatured BSA. 5×10^3 (96 well plates) or 5×10^4 (48 well plates) BC per well will be incubated with purified anti-integrin Mabs (10-20 ug/ml) and allowed to adhere to the coated substrates for 30-60 min at 37° C. Non-adherent cells will be removed by washing and the adherent cells will be stained with crystal violet, solubilized and quantitated with crystal violet as described above (proliferation assay).

f. Preparation of Adhesive Proteins and GRGDS Peptides

pFN, VN and Type I COL will be as previously described (Wayner and Carter, 1987; Wayner et al., 1988; Wayner et al., 1991). GRGDS, GRGES peptides and GRGDS conjugated to rabbit serum albumin (rsa) were synthesized and purified as previously described (Wayner and Kovach, 1992) and were the generous gifts of the Bristol Myers-Squibb Pharmaceutical Research Institute, Seattle, WA.

g. Flow Cytometry Analysis

Flow cytometry analysis will be used to identify the integrin receptors expressed by freshly derived and in some cases cultured normal or transformed BC lines. Cells will be stained with purified anti-integrin Mabs (10 ug/ml) for 30 min in suspension at 4°C in FACS buffer (HBSS supplemented with 1% goat serum and 0.02% sodium azide). At the end of this incubation the cells will be washed and incubated with affinity purified FITC-conjugated goat anti-mouse, 2 ug/ml (Southern Biotechnology). The appropriate isotype matched controls will be included (Pharmingen). Cells will be analyzed by forward light scatter (linear) versus green fluorescence (log). All flow cytometric analyses of stained cells will be performed with a Becton Dickinson FACScan equipped with an argon laser. At least 5,000 events will be analyzed for each anti-integrin antibody and compared to a matched isotype control.

h. Fluorescence Localization of Integrin Receptors in Focal Adhesions

This will be accomplished exactly as previously described (Wayner et al., 1989; Wayner et al., 1991). Briefly, BC will be plated on glass coverslips coated with 10 ug/ml pFN or 2 ug/ml GRGDS-rsa. Adherent cells will be permeabilized with 0.5% Triton X-100 for 5 min, washed and blocked with 5% BSA in PBS. The permeabilized cells will be stained with antibodies to specific receptors or adhesion ligands (60 min RT) washed and incubated with either FITC-conjugated goat anti-mouse (Southern Biotechnology) or rhodamine-conjugated goat-anti rabbit IgG (Southern Biotechnology). Coverslips will be inverted onto glass slides for fluorescence microscopy and interference reflection microscopy with a Zeiss Axioscope. Focal adhesions formed during the attachment of cells to pFN or GRGDS-rsa coated surfaces will be visualized by interference reflection microscopy (IRM, Carter et al., 1990a) or the exclusion of a ligand specific antibody from the close contacts and by vinculin co-localization (Wayner et al., 1991).

i. Immunoprecipitation and Western Blotting

Immune precipitation analysis will be used to evaluate the presence and structure of integrin receptors expressed by BC and to precipitate cyclin and CDK-associated kinase activities. In the first case, immune

precipitation of integrin receptors will be carried out essentially as described (Wayner and Carter, 1987; Wayner et al., 1989; Wayner et al., 1991) except that the BC will be biotinylated to label surface proteins. Immune precipitates will be solubilized in SDS-PAGE buffer and analyzed on 7.5% polyacrylamide slab gels. The electrophoresed proteins will be transferred to nitrocellulose by Western blot techniques (Brown et al., 1991) and the presence of biotinylated protein bands on the nitrocellulose will be detected using HRP-conjugated streptavidin (BioRad) and chemiluminescence as described (Wewer et al., 1994). Immune precipitations to detect cyclin or CDK associated kinase activities will be essentially the same and as described (Symington, 1992). In some experiments, immune precipitations will be carried out in combination with Western blot techniques to identify specific cyclin/cdk complexes. This will be particularly relevant to probe the role of cyclin A in integrin-mediated signaling and to examine the possibility that integrins induce cyclin switching by cdc2 in BC (Aim 1).

j. Kinase Assay

The in vitro kinase assay will be carried out essentially as described (Symington, 1992). Briefly, normal, freshly derived or cultured neoplastic BC will be incubated for 2 hr in RPMI 1640 containing 10 ug/ml GRGDS (or GRGES). In some experiments pFN or anti-α5 and anti-β1 Mabs will be used to ligate α5β1. Activated cells will be solubilized and the cell lysates reacted with anti-cdc2 (Pharmingen clone A-17 precipitates an active kinase), anti-cdk2 or mouse IgG (negative control) followed by protein A-agarose beads. In some experiments associated kinase activities will be precipitated with anti-cyclin Mabs. Protein A beads will be washed and resuspended in kinase buffer (20 mM Tris, 10 mM MgCl$_2$, 1 mM dithiothreitol, 30 μM ATP, 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham, Corp.) with or without 1 μg histone H1 per reaction tube and incubated at 37° C for 30 min prior to solubilization in SDS-PAGE buffer and electrophoresis on 12% polyacrylamide slab gels. Gels will be fixed in methanol/acetic acid, dried and exposed to film. Radiolabeled histone H1 bands will be quantitated by densitometry.

2. Results (Figures 1-6 are on pages 16-18, Table 1 is on page 18)

Aim 1: Identify the mechanism of α5β1-mediated growth stimulation in normal BC and non-transformed (HBL 100) BC lines.

Pitfall Encountered in Aim 1. In the original grant application normal BC were to be compared with HBL 100. We have obtained these cells from the ATCC and are currently growing them in the laboratory. However, HBL 100 cells are propagated in medium containing serum which contains high levels of both fibronectin and vitronectin. Therefore, we are currently establishing a variant cell line which is propagated in serum-free MEGM. Once we have this HBL 100 sub-line we will compare its response to integrin ligands with normal BC. However, we have initiated the studies planned to examine GRGDS-dependent kinase activities in normal BC (**Figures 1-3, page 16**).

a) Examine the ability of integrin ligands to induce proliferation of normal breast epithelial cells (BC) (Figure 1, page 16). The purpose of these experiments was to determine if any integrin ligand could regulate normal BC proliferation. Therefore, the effects of pFN, VN, Type I COL, GRGDS or GRGES on normal BC proliferation were examined. Normal BC were obtained from Clonetics and maintained in MEGM. At passage 3 they were trypsinized and seeded onto 96 well plates and incubated for 24 hrs. At time 0 various integrin ligands (GRGDS, GRGES, pFN, VN, Type I COL) were added at equimolar concentrations (20 uM). At intervals, cells were fixed, stained with crystal violet, solubilized and the plates were read on a Molecular Dynamics plate reader at 540 nm. As can be seen from Figure 1, only pFN or GRGDS could induce proliferation of normal BC (data shown for 5 day time point). VN, Type I COL and GRGES had no effect on primary normal BC proliferation. These results are in agreement with those obtained by the previous PI using normal primary human foreskin keratinocytes and support the hypothesis that ligation of α5β1 provides a positive growth regulatory signal to normal BC. They further suggest that GRGDS can induce a growth promoting signal in G$_1$. This possibility will be investigated by examining phosphorylation of the retinoblastoma protein (Rb) as a function of GRGDS treatment in normal BC (Symington, 1992). These experiments will be performed on normal, transformed (HBL 100) and tumorigenic (BT20) Rb cells.

b) Examine the effects of ligand valence on $\alpha5\beta1$-mediated BC proliferation or kinase activity (Figure 2, page 16). Preliminary data obtained by the previous PI with FA-K562 cells or normal keratinocytes suggested that multivalent interactions with $\alpha5\beta1$ are less effective in stimulating cdc2 activity than low valancy interactions with soluble GRGDS peptides or soluble Mabs. Our preliminary findings support this notion since 20 uM GRGDS was more effective than pFN (20 uM) at stimulating cdc2 kinase activity in normal BC (**Figure 2**). This is an important finding in that GRGDS-mediated cdc2 activation can therefore be distinguished from other integrin-mediated signaling events which require integrin clustering (reviewed by Juliano and Haskell, 1993). The purpose of the experiments in this Aim is to further investigate how ligand valency affects $\alpha5\beta1$ signaling in normal BC. A simple way to increase the valency of integrin interactions is to immobilize the ligands (Schwartz et al., 1991). Therefore, the following ligands will be examined for their ability to induce normal BC proliferation: soluble pFN (two GRGDS sequences), immobilized pFN (multivalent), soluble anti-$\alpha5$ (P1D6) and anti-$\beta1$ (P4C10), or immobilized anti-$\alpha5$ and anti-$\beta1$ (multivalent). In addition, we will also use soluble GRGDS peptides, a multivalent GRGDS-rabbit serum albumin conjugate (Wayner and Kovach et al., 1992) and an 80 kDa proteolytic fragment of fibronectin which contains the central cell binding domain and two GRGDS sequences per molecule (Wayner et al., 1989). The ability of these agents to induce integrin clustering (focal adhesions) in BC will be examined by immunofluorescence and interference reflection microscopy (IRM). I am highly experienced with this technique (Wayner et al., 1989; Carter et al., 1990a, 1990b; Wayner et al., 1991) and have a Zeiss Axioscope equipped with epifluorescence and IRM capabilities in my laboratory. Therefore, I do not anticipate any problems in evaluating $\alpha5\beta1$ distribution in adherent BC populations.

c) Determine the kinetics of cdc2 activation in breast cells (Figure 3, page 16). The purpose of the experiments in this Aim is to determine if ligation of $\alpha5\beta1$ with GRGDS will rapidly activate cdc2. The kinase experiments were carried out exactly as described (Symington, 1992) using a rabbit polyclonal antisera to the unique carboxyl terminus of cdc2 obtained from Pharmingen (cat. #14736E) and 10 ug/ml GRGDS peptide. The results are shown in **Figure 3** and demonstrate clearly that 10 ug/ml GRGDS can rapidly activate cdc2 kinase activity in normal BC as detected by phosphorylation of histone (H1). Since BC rapidly activate cdc2 in response to GRGDS this suggests that GRGDS induced cdc2 activation is not the result of new protein or mRNA synthesis. This possibility will be tested by determining the ability of GRGDS to induce kinase activities in cycloheximide (inhibits protein translation) or actinomycin D (inhibits translation).

d) Determine the concentration dependence of GRGDS mediated cdc2 activation in breast cells (BC). Dose response curves will be obtained for normal, transformed (HBL 100) and tumorigenic (BT20) BC and for each $\alpha5\beta1$ ligand (pFN, soluble GRGDS, multivalent GRGDS-rsa, the 80 kDa fragment, anti-$\alpha5$ or $\beta1$ Mabs).

e) Examine cyclin/CDK switching in BC: Identify cdc2-associated cyclin(s) in GRGDS-treated BC.

i) Monitor cyclin A expression and cdc2 association in normal versus BC lines. The results of the previous PI and those presented here suggest that GRGDS modulates growth responses in FA-K562 cells by triggering the G1/S transition. The results of two recent experiments suggest that cyclin A might be a critical cell cycle dependent protein to monitor as a function of GRGDS treatment. Elledge et al., (1992) have reported that in HeLa cells grown in suspension, cyclin A was associated with cdc2 while in adherent HeLa cells cyclin A was associated with cdk2. In addition, Guadagno et al.(1993) have shown that appearance of cyclin A mRNA and protein in late G_1 was dependent on cell adhesion in NRK and NIH 3T3 fibroblasts. Ectopic expression of cyclin A resulted in adhesion independent accumulation of cyclin A protein and cyclin A-associated kinase activities. In contrast, expression of cdc2, cdk2, cyclin D1 and cyclin E was independent of adhesion suggesting that cyclin A is a target of the adhesion-dependent signals that control proliferation. Therefore, I am particularly enthusiastic to examine the effects of integrin receptors on cdc2-cyclin A association in normal, transformed and tumorigenic BC. We will purchase anti-cyclin A reagents from Pharmingen (cat # #14531C) for use in immunoprecipitation and kinase assays (as described above).

ii) Identify the cdc2-associated cyclin in GRGDS-treated normal BC. Because integrins mediate cell-substrate adhesion in normal cells, it is possible that these receptors can participate in determining cdc2/cyclin associations when cells are switched from an adherent state to a non-adherent state. Preliminary data obtained by the previous PI showed that in FA-K562 cells, GRGDS reduced the level of cdc2 associated with cyclin A without reducing the levels of cdk2 associated with cyclin A suggesting that GRGDS might exert its effect on FA-K562 cells via cyclin/CDK switching. It has been proposed by the previous PI that "transformed BC could escape from α5β1 determined growth constraints because a) they cannot assemble an appropriate cdc2/cyclin complex, b) they constitutively express the "active" cdc2/cyclin complex, or they cannot "switch" cdc2/cyclin partners". To discriminate between these possibilities, the cdc2/cyclin associations in normal BC will be identified in order to determine if GRGDS-treatment can affect cdc2-cyclin associations in normal BC. First, the kinase activity of cyclin immunoprecipitates (Mabs to cyclins A, B1, D1-D3 and E, Pharmingen) will be determined in GRGDS verses GRGES-treated normal BC. The kinase assay will be identical to that described above for cdk immune precipitates. This experiment will provide indirect evidence characterizing cyclin/cdc2 complexes triggered by GRGDS because cyclins have no endogenous kinase activity (reviewed by Cherr, 1994). If GRGDS induces increases in any other cyclin-associated kinase activities this would suggest that GRGDS induces the formation of complexes between cdc2 and these particular cyclins. This possibility will be examined by directly measuring the physical association between cdc2 and the known cyclins. In these experiments lysates of 10^7 control or GRGDS treated normal BC will be immunoprecipitated with anti-cdc2. Cdc2 immunoprecipitates will be electrophoresed, blotted onto nitrocellulose, and probed with anti-cyclin antibodies (Pharmingen, Hot Lines, 2(1): 4, 1995). If GRGDS consistently induces changes in the level of a particular cyclin which is associated with cdc2, this will indicate that GRGDS is capable of inducing cyclin switching in normal BC. If no increases in kinase activity associated with cyclins A-E are found it will suggest that GRGDS induces the association of cdc2 with a novel cyclin. This new cdc2 partner will be identified using standard monoclonal antibody technology and protein biochemistry techniques. In addition, to my experience in making Mabs to cell surface receptors, I have also recently made Mabs to the *S. cerevisiae* transcription factors, SWI4 and SWI6 (in collaboration with Dr. Linda Breeden, FHCRC, Seattle, WA) and a nuclear transcription factor, PIE 1, expressed in *C. elegans* (in collaboration with Dr. James Priess, FHCRC, Seattle, WA). Therefore, I do not anticipate any problems in identifying potential new cyclins complexed with cdc2 in GRGDS treated BC.

Aim 2: Examine the effect of GRGDS on growth and cdc2 activation in immortalized (transformed) human BC lines and identify the cause(s) underlying the abnormal growth response to GRGDS.

Pitfall Encountered in Aim 2: We have access to a number of transformed breast cell lines including those described by the previous PI, either through my own previous work (Wayner et al., 1989; Brown et al., 1991) or from the ATCC. Although these studies were not described in the grant application written by the previous PI, we have decided to examine the expression and function of integrins receptors in normal and transformed BC populations for the following reasons. Several integrin receptors including αvβ3 and αvβ5, as well as α5β1 can interact with FN (αvβ3) and RGD-containing peptides (αvβ3 and αvβ5) (reviewed by Fielding-Habermann and Cheresh, 1993). In cell populations which express both αvβ3 and α5β1, these receptors cooperate to mediate adhesion to fibronectin (Charo et al., 1990). Although this seems paradoxical, it has generally been found that when cells express multiple receptors to an adhesive protein, adhesion to the protein is usually accomplished via the simultaneous engagement of both receptors (Wayner et al., 1989; Wayner et al., 1991). Normal breast epithelial cells express low levels of αvβ5 and are negative for αvβ3, and appear to use only α5β1 to adhere to pFN (**Figures 4 and 5, page 17**). In contrast, we have found that several transformed BC lines (e. g. BT20) express high levels of αvβ3 (**Figure 5, page 17**) and use both α5β1 and αvβ3 to bind FN or GRGDS-rsa coated surfaces (**Figure 6, page 18**). This presents a problem due to the potential effects of αvβ3-mediated signaling as a function of binding to GRGDS or pFN. Therefore, we have altered Aim 2 (and Aim 3) to include a new technical objective the purpose of which will be to screen all BC populations for the presence and functional activity of alternative FN receptors.

a) **Determine the integrin phenotype of transformed BC lines.** The integrin phenotype of normal or transformed BC lines obtained from the ATCC will be examined by flow cytometry and immune precipitation analysis. I am highly experienced with both of these techniques (Wayner et al., 1988; 1989;

1991; 1993) and do not anticipate any problems in determining the presence of alternative functional FN receptors expressed by BC populations. We have initiated these experiments and the results of our preliminary studies are summarized in **Table 1 (page 18)**. If possible, we plan to establish variant cell lines derived from each of these populations which will be propagated in serum-free MEGM. This will minimize the effects of constantly selecting cell populations for growth in serum containing media which contains both fibronectin and vitronectin (serum spreading factor) (see reviews by Ruoslahti, 1988; Fielding-Habermann and Cheresh, 1993). Alternatively, we can select cell lines by fluorescence activated cell sorting ($\alpha5\beta1+$, $\alpha v\beta3$ -) or plating cells on surfaces coated with anti-$\alpha5$.

b) Examine growth responses to GRGDS in $\alpha v\beta3$ negative transformed and immortalized BC lines.

c) Measure GRGDS affinities of normal and transformed $\alpha v\beta3$ negative BC.

d) Examine surface $\alpha5\beta1$ expression structure and distribution in transformed $\alpha v\beta3$ negative BC lines.

e) Identify differences in post-ligand binding events in normal versus transformed $\alpha v\beta3$ negative BC.

Aim 3: Examine the effect of GRGDS on growth and cdc2 activation in freshly isolated neoplastic BC and identify the cause(s) underlying abnormal growth responses to GRGDS.

Pitfall Encountered in Aim 3. We have obtained two tumor specimens from Dr. Tickman. The tumor specimens are very small (less than 1 cm) and due to the use of mammography and other early detection techniques (breast examination) many future tumors are likely to be less than 1 cm. Therefore, we will need to investigate the possibility that we can establish primary cell lines from breast tumors in order to accomplish this aim. Although there are sound reasons for using freshly derived cells, it may not be possible to acquire enough cells from every tumor to accomplish the analyses. Establishing cell lines from primary tissue is fraught with difficulties, one of the primary problems being that the cells are grown in serum and only cells which are capable of interacting with serum factors (FN or VN) are selected. Therefore, we plan to establish primary breast carcinoma cell lines in MEGM to avoid the issue of plasma FN and VN "selection". "Normal" BC will also be isolated form "normal tissue" adjacent to the tumor involved areas. Second, in order to be sure that the integrin phenotype of the resulting cell lines has not been altered by the culture conditions we will examine the expression of integrin receptors in the breast tissue by immunohistochemical (IH) techniques. I am an expert in the localization of integrin receptors in frozen tissue specimens by IH techniques (Hoffstrom and Wayner 1995) and my laboratory is equipped with an IEC cryostat. Therefore, we do not anticipate any problems in phenotyping the breast carcinomas by IH techniques. With large tissue pieces (>1 cm) we will isolate neoplastic and normal breast epithelial cells in order to carry out the experiments in this Aim as described by the previous PI.

a) The integrin phenotype of primary BC populations will be determined by fluorescence (see above) and $\alpha v\beta3$ negative populations will be selected for further analysis.

b) The effects of GRGDS and $\alpha5\beta1$ ligation on proliferation, CDK and cyclin activity will be examined in freshly isolated neoplastic breast cells and compared to normal BC.

c) Determine if progressive defects in $\alpha5\beta1$ signaling accompanies progressive steps in neoplastic transformation (tumor progression and tendency to metastasize).
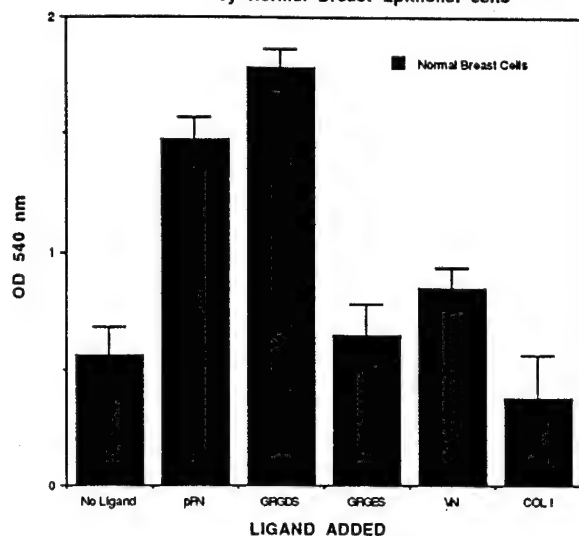
**pFN or GRGDS Peptides Stimulate Proliferation
by Normal Breast Epithelial cells**

Figure 1. Effect of integrin ligands on proliferation of normal breast epithelial cells. Integrin ligands (20 uM) were added at the initiation of culture and the data shown was derived five days post addition of the ligands. Cell numbers were estimated by staining with crystal violet as described (Experimental methods).
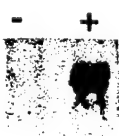
Efficacy of GRGDS or pFN to Induce Cdc2 Kinase Activity in Normal Breast Cells

GRGDS (10 uM):   -   +

pFN (10 uM):   -   +

Figure 2. pFN is less effective than GRGDS peptide in activating cdc2 kinase activity in normal BC. In these experiments normal BC were activated with 10 uM GRGDS (+) or pFN (+) for 60 min. Kinase assays were performed as described (Symington, 1992) using histone H1 as a substrate for immune precipitated cdc2.

Kinetics of Cdc2 Activation by GRGDS

In Normal Breast Cells

GRGDS (10 ug/ml):

GRGES (10 ug/ml):

      1 2 3 4 5 6

Figure 3. Kinetics of Cdc2 kinase activity in normal BC stimulated by GRGDS peptide (10 ug/ml). Kinase activity was detected by phosphorylation of H1 histone. Lane 1 (0 min), lane 2 (5 min), lane 3 (15 min), lane 4 (60 min), lane 5 (180 min), lane 6 (270 min). GRGDS stimulated cdc2 kinase activity at the earliest time point examined (15 min) and continued to rise throughout the incubation period.

Inhibition of Normal BC Adhesion to pFN by Mab P1D6 (anti-α5)

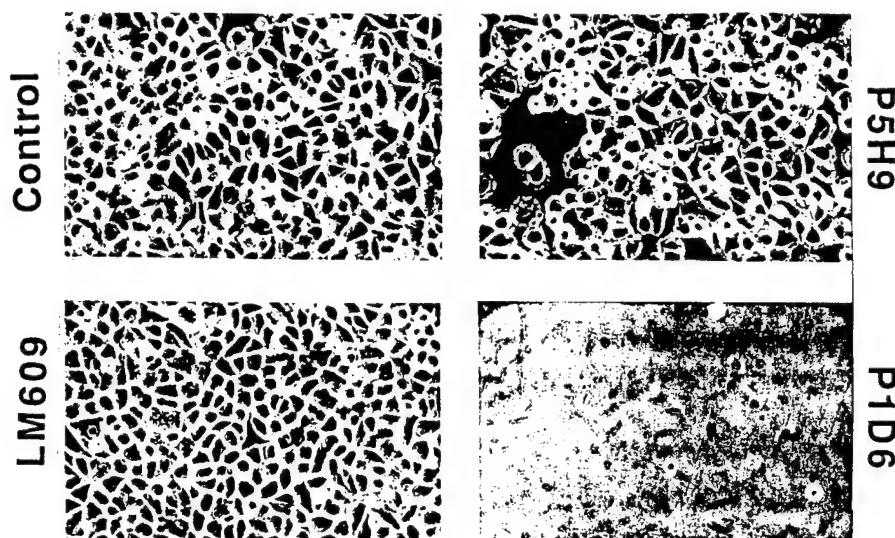# but not by Mab LM609 (anti-αvβ3) or Mab P5H9 (anti-αvβ3)

Figure 4. Inhibition of normal BC adhesion to pFN-coated surfaces (10 ug/ml coating concentration) by Mabs (10 ug/ml) directed to known FN integrin receptors. Control = purified mouse IgG (10 ug/ml).

Detection of αvβ3 in BT 20 Cells But Not Normal BC
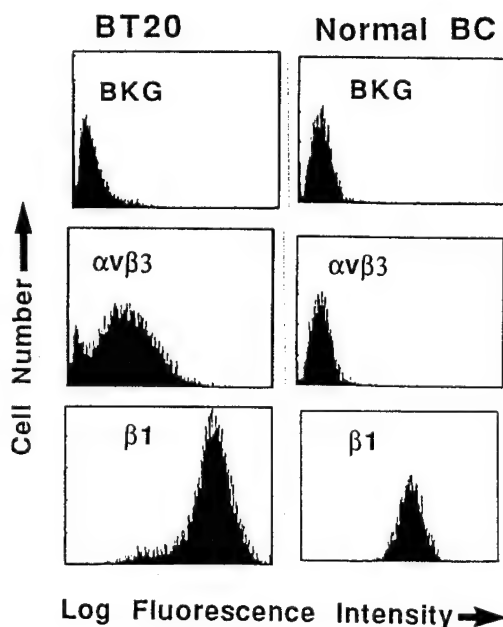
Cells by Flow Cytometry

Figure 5. Detection of αvβ3 or β1 in BT20 but not normal breast epithelial cells by staining with Mabs LM609 (αvβ3) or P4C10 (β1) and flow cytometric analysis.
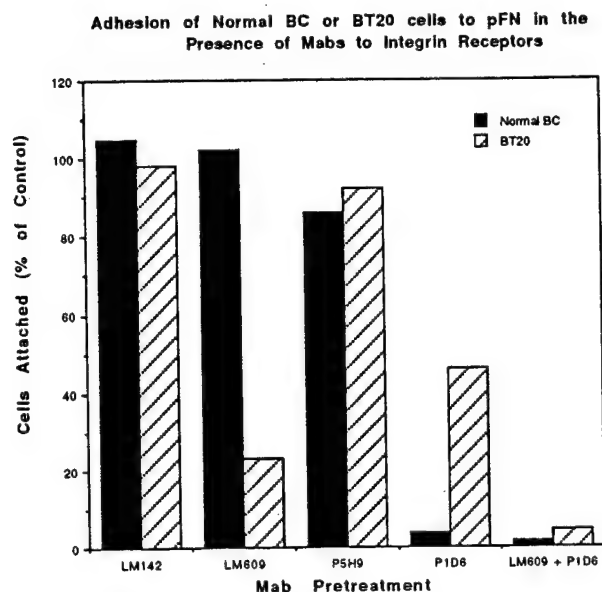
Figure 6. Inhibition of normal or BT20 breast cell adhesion to surfaces coated with pFN (10 ug/ml coating concentration) with Mabs (10 ug/ml) directed to integrin receptors. The following purified anti-integrin Mabs were used: LM142 (anti-αv, courtesy of D. Cheresh, Scripp's Clinic), LM609 (anti-αvβ3, courtesy of Dr. Cheresh), P5H9 (anti-αvβ5, Wayner et al., 1991), and P1D6 (anti-α5, Wayner et al., 1988).

Table 1: Expression and Function of the Integrin Receptors

α5β1 and αvβ3 Expressed by BC Populations

Cell Population	Transformation Status	α5β1	αvβ3	Recptor Used to Bind FN
Primary BC	Normal	+/-	-	α5β1
HBL 100[a]	Transformed	+	-	α5β1
BT20[a]	Tumorigenic	+	+	α5β1, αvβ3
T-47D[a]	Transformed	+	-	
Hs 578Bst	Abnormal			
Hs 578T	Tumorigenic			
MDA-MB-157	Tumorigenic			
MDA-MB-453	Transformed			
MDA-MB-361	Metastatic			
DU4475	Metastatic			
BT-483	Tumorigenic			

[a]Charateristics determined on cells grown in RPMI supplemented with serum.

C. CONCLUSIONS

In summary, our preliminary findings suggest the following:

1. That primary breast cells rapidly activate cdc2 in response to ligation of α5β1 with GRGDS peptides. This implies that α5β1 is involved in normal growth control in breast epithelial cells. We will continue with the studies in Aim 1 as described by the previous PI. In addition, since other CDKs are known to participate in checkpoints throughout the cell cycle, we will also examine whether α5β1 can participate in the activation of any other CDK. The previous PI has shown that in FA-K562 cells cdk2 was not activated by GRGDS. However, at the present time we do not know whether this will be the same for BC. Mabs to other CDKs will be purchased from Pharmingen and the effects of GRGDS will be examined using immune precipitation and phosphorylation of histone as an indicator of GRGDS-induced alternative CDK activity.

2. Long term cultured breast cells lines such as are available from the ATCC were originally selected in medium containing serum and are continuously propagated in medium containing serum (see ATCC Catalogue) and therefore many of these cell populations will express alternative functional fibronectin receptors (e. g. αvβ3). This implies that our analysis of cultured cell lines will be complicated by the presence of αvβ3 and potential growth signaling capabilities of this receptor. In order to circumvent this problem we will take two approaches. First, all BC populations will be screened for the expression and function of alternative fibronectin receptors. Second, we will attempt to generate variant α5β1+ and αvβ3- cell lines by fluorescence activated cell sorting or growth in serum free MEGM. Finally, if time and funds permit we may investigate potential signaling mechanisms and growth control mediated by αvβ3 expressed in transformed or tumorigenic BC population.

3. Many of the tumor specimens provided by Dr. Tickman will be too small to isolate large numbers of epithelial cells for in vitro analyses. Therefore, we will attempt to generate primary cell lines in serum free MEGM for use in the studies described in Aim 3. All of the tumor specimens will be phenotyped by IH techniques to determine if our culture conditions induce de novo expression of alternative FN receptors.

4. In addition, although this issue was not addressed by the previous PI, it is possible that another mechanism of integrin mediated growth control in normal BC is the induction of specific cyclin-CDK inhibitors such as p16, p21 or p27. Therefore, it is possible that neoplastic BC escape from the normal growth constraints by failing to couple integrin-mediated signaling events with CDI induction. This possibility will be investigated by examining CDI induction via ligation of integrin receptors in normal BC. If a specific interaction is observed to occur between an integrin receptor and induction of a CDI, this mechanism of growth control will be investigated in transformed BC populations.

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E. APPENDIX

Content of the Appendix (following)

Curriculum Vitae for Dr. Tom Burkett

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Frederick, MD 21701

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Seattle, WA. 98109
206-285-8678

EXPERIENCE:

Staff Scientist, Proteinix Co. (IGEN, INC.)   6/94-9/95
16020 Industrial Drive
Gaithersburg, MD 20877
(301) 984-8000 (x1088)

Responsible for the cloning, expression and optimization of recombinant protein
production in yeast (*Saccharomyces cerevisiae, Pichia pastoris*) and bacteria (*Esherichia
coli*). In this position I cloned and produced peptides using ubiquitin fusion technology.
In addition, I expressed recombinant antibody-like molecules in yeast and bacteria,
developed vectors for expression of antibody-like molecules in yeast, and cloned antigens
from cDNA libraries.

Postdoctoral Fellow, Movable Genetic Elements Section   9/93-6/94
Laboratory of Eukaryotic Gene Expression
National Cancer Institute-Basic Research Program
Frederick, MD
Used classical and molecular genetic techniques to characterize interactions between
genetic suppressors of transposable element induced mutations (The *SPT23* gene) and
other genes (*MGA2*) in *Saccharomyces cerevisiae* .

Predoctoral Fellow, Movable Genetic Elements Section   9/88-9/93
Laboratory of Eukaryotic Gene Expression
National Cancer Institute-Basic Research Program
Frederick, MD
Used Classical and molecular genetic techniques to isolate and characterize genetic
suppressors of transposable element induced mutations in *Sacharomyces cerevisiae.*

Masters Student, University of Maryland Baltimore County   9/86-6/88
Catonsville, MD
My thesis topic focused on the identification and cloning of genes encoding
photosynthetic proteins from a marine alga. In addition, I developed and assayed
monoclonal antibodies against surface proteins of the marine gliding bacterium
Flexibacter maritimus.

Analytical Chemist
Western Technologies Inc.   7/85-9/86
Phoenix, AZ
Performed analysis of trace metals and organics in environmental samples for an
environmental engineering firm.

Analytical Chemist
Bolin Laboratories Inc.   11/84-7/85
Phoenix, AZ
Performed instrumental and wet chemical analysis of food, water and pharmaceutical
samples using official A.O.A.C. methods.

EDUCATION:
Ph.D., Microbiology   8/93
University of Maryland at College Park
College Park, MD
Dissertation Title: Characterization of the *SPT23* gene: a multi-copy suppressor of
transposable element induced mutations in *Saccharomyces cerevisiae*.

M.S., Applied Molecular Biology   6/88
University of Maryland Baltimore County
Baltimore, MD
Thesis title: Cloning of the *PSBA1* gene from the marine red alga *Porphryidium
cruentum*.

B.S., Chemistry/Biological Sciences   6/84
Western Washington University
Bellingham, WA

MILITARY EXPERIENCE: 6/76-6/79
Graduate of Ranger, Pathfinder, and Jumpmaster schools. Jumpmaster and Pathfinder
on company parachute drops. Numerous awards, distinctions and citations.

TEACHING EXPERIENCE:
Introductory Chemistry Lecturer (Frederick Community College)
Introductory Biology Laboratory Instructor (Frederick Community College)
Sailing Instructor (National Institutes of Health, Parklawn Sailing Association, WWU)

PUBLICATIONS:
Sharon, G.; Burkett, T. J.; and Garfinkel, D. J. (1994). Efficient homologous
recombination of Ty1 element cDNA when integration is blocked. Mol. Cell. Bio. **14:**
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